

# A Mouse Model of *Salmonella* Typhi Infection

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## SUMMARY

*Salmonella* spp. are gram-negative flagellated bacteria that can cause food- and waterborne gastroenteritis and typhoid fever in humans. We now report that flagellin from *Salmonella* spp. is recognized in mouse intestine by Toll-like receptor 11 (TLR11). Absence of TLR11 renders mice more susceptible to infection by *S. Typhimurium*, with increased dissemination of the bacteria and enhanced lethality. Unlike *S. Typhimurium*, *S. Typhi*, a human obligatory pathogen that causes typhoid fever, is normally unable to infect mice. TLR11 is expressed in mice, but not in humans, and remarkably, we find that *tlr11*<sup>-/-</sup> mice are efficiently infected with orally administered *S. Typhi*. We also find that *tlr11*<sup>-/-</sup> mice can be immunized against *S. Typhi*. Therefore, *tlr11*<sup>-/-</sup> mice represent a small-animal model for the study of the immune response to *S. Typhi* and for the development of vaccines against this important human pathogen.

## INTRODUCTION

Innate immune responses provide the first line of host defense against microbial pathogens and, together with adaptive immunity, ensure effective clearance of invading pathogens. Toll-like receptors (TLRs), which are expressed in sentinel cells such as macrophages and dendritic cells, recognize conserved components of microbes known as pathogen-associated molecular patterns (PAMPs) (Kawai and Akira, 2010). Mice express 12 TLRs, including TLRs 11, 12, and 13, which are not expressed in humans (Roach et al., 2005). We have previously characterized the role of the mouse-specific TLR11 in response to uropathogenic *E. coli* and the apicomplexan parasite *Toxoplasma gondii* (Yarovinsky et al., 2005; Zhang et al., 2004). The

*T. gondii* PAMP recognized by TLR11 is the apicomplexan profilin protein (Yarovinsky et al., 2005). The finding that TLR11 recognized a protein ligand from *T. gondii* was consistent with phylogenetic analyses demonstrating that TLR11 is most closely related to TLR5 (Roach et al., 2005; Zhang et al., 2004), which recognizes the bacterial protein PAMP, flagellin (Hayashi et al., 2001).

In contrast to *T. gondii*, the ligand from uropathogenic *E. coli* (UPECs) that triggers innate responses through TLR11 in the urogenital tract has remained uncharacterized (Zhang et al., 2004). Our initial efforts in purifying this ligand suggested that it was a protein component of UPEC, although we were not able to identify the specific molecule recognized by TLR11 (Zhang et al., 2004). Given that PAMPs are rarely restricted to specific pathogens (Kawai and Akira, 2010), we chose to investigate the potential role of TLR11 in recognizing other pathogens in order to identify another source from which we could potentially purify the bacterial ligand for TLR11. Consistent with TLR11 mediating the innate response to UPECs at the urogenital epithelium, we noted that TLR11 is also highly expressed in the intestinal epithelium. This led us to speculate that TLR11 might also function in the recognition of enteropathogenic bacteria.

Gram-negative *Salmonella* sp. is a common bacterial enteropathogen and is widely used in laboratory studies aimed toward understanding the basis of mucosal immune responses and diseases such as gastroenteritis and typhoid. Most laboratory studies are carried out using *S. Typhimurium* in mice, where a disseminated infection with some similarities to human typhoid is observed. Typhoid fever affects more than 20 million individuals and causes more than 220,000 deaths annually (Crump et al., 2004; Woc-Colburn and Bobak, 2009). However, typhoid disease in humans is caused by the specific *Salmonella enterica* serovars Typhi and, to a lesser extent, Paratyphi (Crump et al., 2004), which do not infect mice. There are marked genetic differences between *S. Typhimurium* and *S. Typhi*, which presumably account for the divergent disease states caused by these organisms in people (Sabbagh et al., 2010). As a result, animal models of *S. Typhi* infection are of significant interest for understanding

the biology of this important pathogen. Typhoid is endemic in third world countries, where it has been difficult to achieve adequate improvements in sanitation and water supply to have a timely impact on the disease. Clearly, what is required are effective vaccination strategies. Unfortunately, the two approved vaccines have only modest efficacy (50%–80% protection) (Guzman et al., 2006). The development of better vaccination would require a more complete understanding of the immune response to bacteria as well as the availability of experimentally tractable small-animal models that can be infected by *S. Typhi*.

In this study, we describe the contribution of TLR11 in *Salmonella* spp. infection of the mouse. We have found that TLR11-deficient mice are severely compromised in innate epithelial responses to *S. Typhimurium* and exhibit poorly controlled and widely disseminated infection, resulting in enhanced lethality. We have purified the ligand from *S. Typhimurium* and found that, like TLR5, TLR11 recognizes flagellin. Flagellin is also the TLR11 ligand from UPECs. Flagellin binds TLR11 and induces innate responses in mice independent of TLR5. Remarkably, loss of TLR5 results in upregulation of TLR11 in lamina propria macrophages, suggesting that enhanced TLR11 responses may contribute to the resistance of TLR5-deficient mice to *S. Typhimurium* infection. Because TLR11 contributes significantly to protection of mice from *S. Typhimurium* and the presence of TLR11 correlates with the host restriction of typhoidal *Salmonella* infection, we tested whether TLR11 might be responsible for mouse resistance to *S. Typhi* infection. As for *S. Typhimurium*, we found that TLR11 recognized *S. Typhi* flagellin and that *S. Typhi* could lethally infect mice lacking TLR11 upon oral infection. Low-dose oral infection with *S. Typhi* produced a febrile illness with features of human typhoid. Existing small-animal models of *S. Typhi* infection rely on use of severely immunocompromised animals (Mian et al., 2011) and, therefore, cannot serve as systems in which to study host responses or for vaccine development. In contrast, TLR11-deficient mice can be protected from infection by vaccination with heat-killed *S. Typhi* or by passive transfer of serum from immunized mice. Therefore, these results provide genetic evidence of host restriction enforced by species-specific TLR expression and the generation of an immunologically intact model for assessing *S. Typhi* host-pathogen interactions and testing new vaccine approaches.

## RESULTS

### Lack of TLR11 Leads to Enhanced Susceptibility to Infection by *S. Typhimurium*

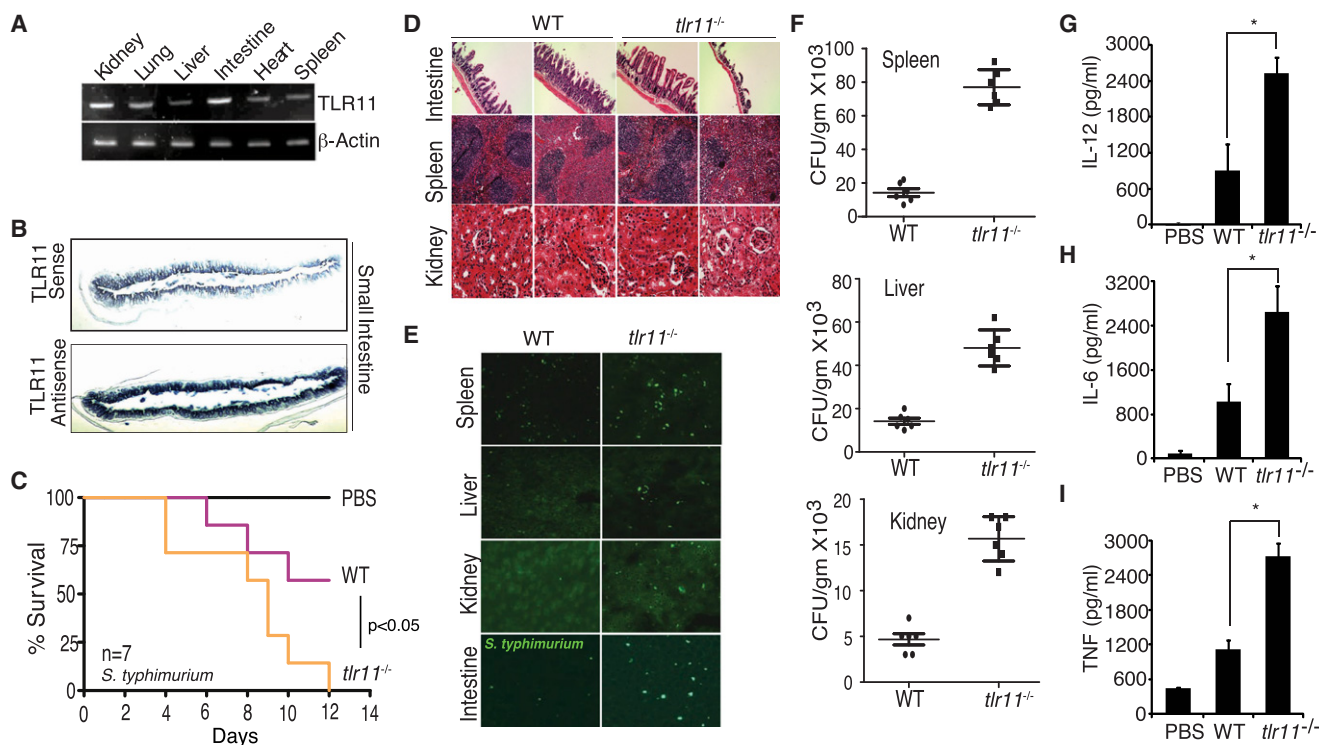
Although we had previously suggested that the PAMP from UPECs recognized by TLR11 was a protein (Zhang et al., 2004), our initial efforts to identify the ligand were unfruitful. We suspected that TLR11 was likely to recognize similar ligands in other pathogenic bacteria, and to help identify additional pathogens that might trigger TLR11-dependent responses, we examined the tissue distribution of TLR11. We carried out RT-PCR to examine TLR11 expression in liver, spleen, kidney, heart, and intestine and observed high relative expression of TLR11 in the small intestine (Figure 1A and Figure S1A available online). We examined the expression of TLR11 in sections from the small

intestine by using in situ hybridization. Similar to the pattern of expression seen in the urinary bladder, TLR11 messenger RNA (mRNA) was strongly enriched in the epithelial layer of the small intestine (Figures 1B and S1B).

The intestinal localization of TLR11 suggested that TLR11 might recognize enteropathogens. As we had established recognition of *E. coli* spp. by TLR11 (Zhang et al., 2004), we wished to determine whether other well-known intestinal pathogens might be recognized by TLR11. *Salmonella* spp. are medically relevant enteropathogenic bacteria, and *S. Typhimurium* is widely used in mouse studies. Given that TLR11 was expressed at the small intestinal epithelium through which *S. Typhimurium* gains entry to the host and that it was also expressed in the macrophage populations in which *S. Typhimurium* is found, we tested whether TLR11 was important for immune responses to orally administered *S. Typhimurium*, to which mice are susceptible. As reported previously, oral administration of high doses of *S. Typhimurium* led to infection and lethality in wild-type mice; however, the lethality was significantly enhanced in *tlr11*<sup>−/−</sup> mice (Figure 1C). Histological studies demonstrated enhanced destruction of the intestinal wall in mice lacking TLR11, which is consistent with an important role in epithelial immunity (Figure 1D). By using *S. Typhimurium* expressing green fluorescent protein (GFP), we were able to observe increased bacterial dissemination that spread beyond the severely damaged intestinal wall in *tlr11*<sup>−/−</sup> mice (Figure 1E). Significantly increased recovery of viable *S. Typhimurium* from the liver, spleen, and kidney of *tlr11*<sup>−/−</sup> mice confirmed the enhanced dissemination in the absence of TLR11 (Figure 1F). Identical results, including intestinal damage and dissemination, were seen with non-GFP-expressing *S. Typhimurium* (Figure S1C). Analyses of serum cytokine levels revealed elevated IL-12 (Figure 1G), IL-6 (Figure 1H), and TNF (Figure 1I) in *tlr11*<sup>−/−</sup> mice, suggesting that bacterial dissemination causes death of the animals by triggering a cytokine storm.

### Flagellin Is a Ligand for TLR11

The TLR11 stimulatory activity found in lysates of the UPEC strain 8NU has a protein component, as this activity is abolished by treatment with proteinase K, but not RNase, DNase, or concanavalin A (ConA) (Zhang et al., 2004). Based on this finding, we speculated that the TLR11 PAMP in *Salmonella* also had a protein component that could be isolated by column chromatography. We fractionated heat-killed lysates of *S. Typhimurium* on a DEAE-Sepharose anion-exchange column, followed by CM-sepharose cation exchange chromatography and finally Mono Q FPLC chromatography using linear NaCl gradients (Figure 2A). The stimulatory activity of dialyzed fractions from each chromatographic step was tracked by testing induction of luciferase in RAW cells, which express TLR11, stably transfected with an NF- $\kappa$ B-luciferase reporter. The stimulatory activity eluted broadly and corresponded to the two major protein peaks (fraction 8 and fraction 14) following Mono Q fractionation (Figure 2B). To confirm that the stimulatory activity in these fractions was of a similar nature to the TLR11 PAMP, we treated fraction 8 and fraction 14 with DNase, RNase, and proteinase K. As lipopolysaccharide (LPS) is a major PAMP in gram-negative bacteria, we also depleted LPS by



**Figure 1. TLR11 Is Expressed in Intestine and Protects against *S. Typhimurium***

(A) RT-PCR analysis of TLR11 in various organs.

(B) In situ hybridization staining (antisense) for TLR11 in small-intestine tissue section.

(C) Survival of wt and *tlr11*<sup>-/-</sup> mice (n = 7) orally infected with *S. Typhimurium* (10<sup>8</sup> CFU).

(D) Tissue damage during *S. Typhimurium* infection shown by H&E staining in small intestine, spleen, and kidney on day 5 postinfection.

(E) Intestinal invasion of *S. Typhimurium* expressing GFP.

(F) Dissemination of GFP *S. Typhimurium* in orally infected WT or *tlr11*<sup>-/-</sup> mice was detected on day 5 postinfection by measuring CFU per gram of spleen, liver, and kidney.

(G-I) Day 5 serum cytokines from WT or *tlr11*<sup>-/-</sup> mice orally infected with *S. Typhimurium* measured by ELISA. \*p < 0.001.

Related to Figure S1.

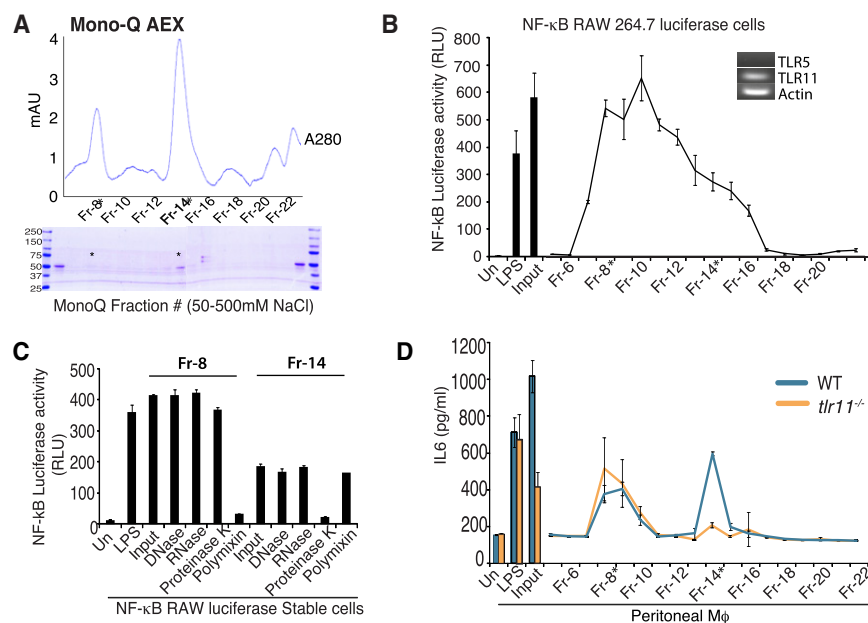
using polymyxin B sepharose. As shown in Figure 2C, the NF-κB stimulatory activity in fraction 8 was unaffected by DNase, RNase, and proteinase K, but was severely diminished by polymyxin B treatment, and hence, the activity in fraction 8 is likely due to LPS. In contrast, the stimulatory activity in fraction 14 was unaffected by DNase, RNase, and polymyxin B but was abolished by proteinase K treatment, strongly suggesting a protein PAMP.

To test the specificity of these fractions for TLR11, we measured the induction of IL6 following stimulation of peritoneal macrophages from either wild-type (WT) or *tlr11*<sup>-/-</sup> mice. The stimulatory activity in fraction 8 was unaffected by the absence of TLR11; however, the ability of fraction 14 to stimulate IL6 production was completely abolished in the *tlr11*<sup>-/-</sup> cells (Figure 2D) or upon treatment with proteinase K (Figure S2A). Therefore, fraction 14 contains a *Salmonella*-derived protein with TLR11-dependent stimulatory activity. SDS-PAGE analysis of fraction 14 revealed few protein bands but included a prominent band around 50 kDa with an elution pattern that matched the TLR11-dependent activity (Figures 2A and 2B). We excised that band and analyzed it by mass spectrometry, and surpris-

ingly, the only protein detected was *S. Typhimurium* flagellin (Figure S2B).

Although the stimulatory activity observed in fraction 14 was dependent upon the expression of TLR11 (Figures 2D and S2A), we were concerned that stimulation might occur through the known flagellin receptor, TLR5. However, consistent with previous reports (West et al., 2005), we found that the mouse macrophage cell line RAW 264.7 does not express TLR5 (Figure 2B, inset), suggesting that the stimulation by fraction 14 (and hence flagellin) was not due to TLR5. The recognition of *Salmonella* flagellin by TLR5 is believed to be important for mounting of innate responses to this bacteria (Fournier et al., 2009; Uematsu et al., 2006), although the role of TLR5 is not straightforward; mice lacking TLR5 show enhanced resistance to *S. Typhimurium* (Uematsu et al., 2006). Our results suggested that TLR11 might play a more significant role in murine resistance to *S. Typhimurium*.

Our previous results had demonstrated that UPEC also possess a proteinase-K-sensitive ligand recognized by TLR11. We therefore carried out an independent purification similar to that used for *S. Typhimurium*. The peak, proteinase-K-sensitive,



**Figure 2. TLR11 Recognizes *S. Typhimurium* Flagellin**

(A) The TLR11 ligand was purified by mechanical disruption of *S. Typhimurium* followed by sequential DEAE-sephacel and CM-Sephacel chromatography. Fractions with stimulatory activity in Raw- $\kappa$ B-luc cells were further subjected to MonoQ FPLC with a linear NaCl gradient. Shown is absorbance at 280 nm over the course of the elution. The fractions indicated were resolved by SDS PAGE, and proteins were visualized by Coomassie staining.

(B) Stimulatory activity of MonoQ fractions was assessed in Raw- $\kappa$ B-luc cells.

(C) Fractions 8 and 14 were subjected to DNase, RNase, Proteinase K, and Polymyxin B treatment and assessed for stimulatory activity in Raw- $\kappa$ B-luc cells.

(D) Peritoneal macrophages from WT or *tlr11*<sup>-/-</sup> mice were stimulated with Mono-Q fractions, and IL-6 production was analyzed by ELISA.

Related to Figure S2.

TLR11-stimulatory fraction from Mono Q column chromatography of 8NU lysates was sequenced by mass spectrometry and yielded *E. coli* flagellin as the primary component (Figures S2C–S2E). Therefore, TLR11, which is highly enriched at epithelial surfaces, including bladder (Zhang et al., 2004), kidney, and small intestine (Figure S1A), is capable of recognizing flagellin from enteropathogenic and uropathogenic bacteria. Taken with the enhanced susceptibility of TLR11-deficient mice to *S. Typhimurium* and UPEC infection, these data suggest nonredundant roles for TLR5 and TLR11 in the recognition of flagellated bacteria.

### Recognition of Flagellin by TLR5 and TLR11

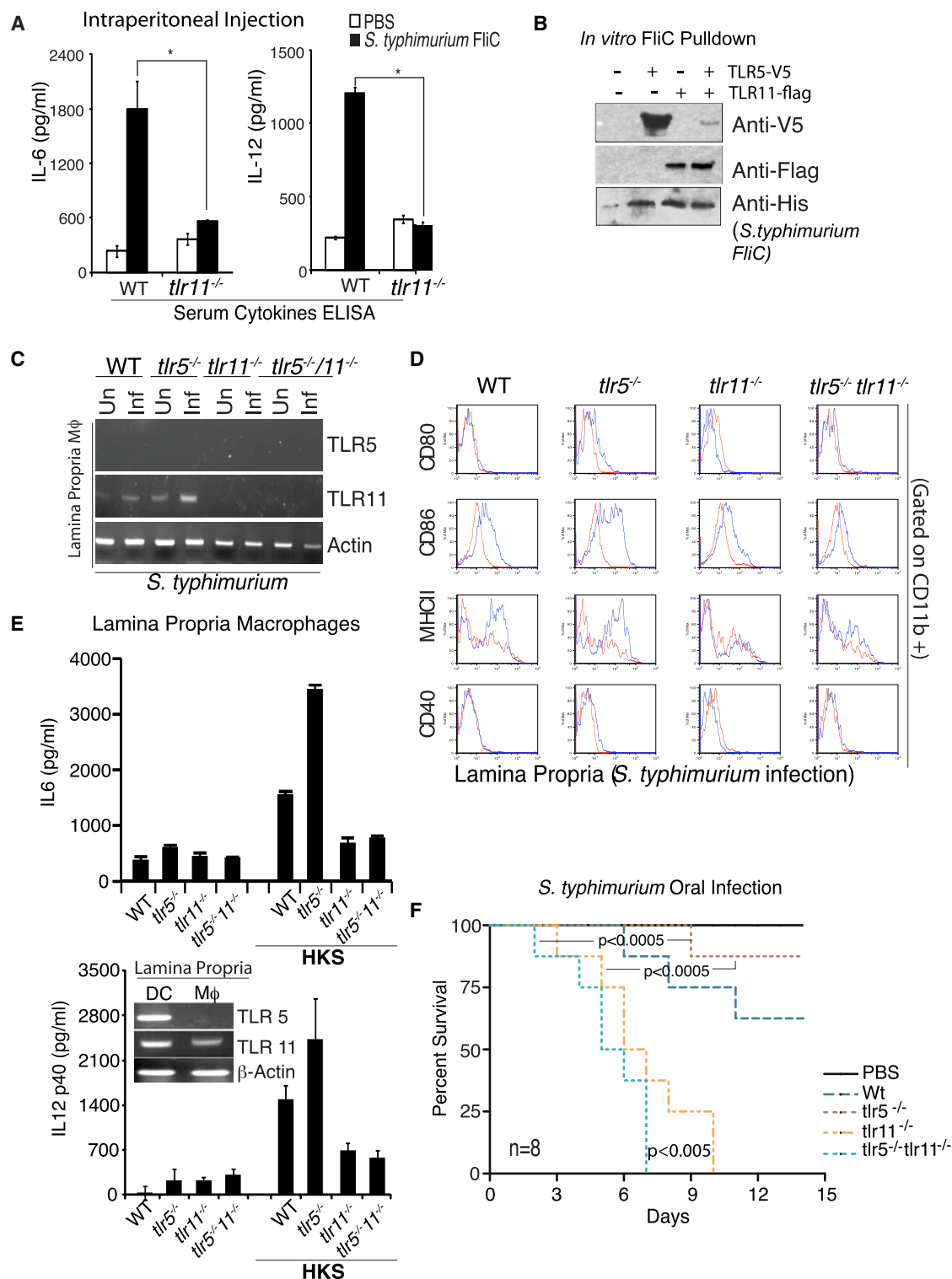
To directly examine whether flagellin responses depend on TLR11, we purified recombinant *S. Typhimurium* flagellin to homogeneity, followed by passage through polymyxin B sepharose resin to remove contaminating LPS. We injected this purified flagellin into WT and *tlr11*<sup>-/-</sup> mice and measured serum levels of IL-6 and IL-12. TLR11 knockout (KO) mice produced significantly less IL-6 and IL-12 following injection of recombinant flagellin (Figure 3A). Consistent with previous reports (Feuillet et al., 2006; Uematsu et al., 2006), production of serum IL-6 was also partially reduced when flagellin was injected into TLR5 KO animals (data not shown), suggesting that both can independently recognize flagellin.

To further explore the role of TLR5 and TLR11 in the recognition of *S. Typhimurium* flagellin, we generated C-terminally epitope-tagged versions of TLR5 (TLR5-V5) and TLR11 (TLR11-Flag). Using lysates from HEK293 T cells expressing TLR5, TLR11, or both, we performed pull-downs with recombinant polyhistidine-tagged *S. Typhimurium* flagellin. Flagellin was captured by using nickel beads, and binding of TLR5 and TLR11 was assessed by immunoblotting. Consistent with the ability of TLR5 and TLR11 to activate cytokine expression in response to flagellin, both TLRs were capable of interacting

with flagellin (Figure 3B). To examine whether TLR5 and TLR11 might interact with each other and hence act as a heterodimer in cells in which they are coexpressed, we mixed Flag-tagged TLR11 with V5-tagged TLR5 and HA-tagged TLR11. Interestingly, TLR11 can form a heterodimer with TLR5 but can also form homodimers (Figure S3A).

We had been intrigued by the increased resistance of mice lacking TLR5 to infection by *S. Typhimurium* (Uematsu et al., 2006) and wondered whether TLR11, given that it could signal in macrophages lacking TLR5, might have a role in this resistance. Different explanations have been advanced to explain this phenomenon, including the possible influence of altered commensal bacterial flora and enhanced IL-1 $\beta$  activity in the TLR5 KOs (Carvalho et al., 2011; Vijay-Kumar et al., 2008). In addition to epithelial cells, sentinel cells of the intestine include lamina propria (LP), macrophages (M $\phi$ s), and dendritic cells (DCs). We therefore analyzed TLR5 and TLR11 mRNA expression in these two cell types. Whereas LP-DCs express both TLR5 and TLR11, we found that LP-M $\phi$ s express TLR11, but not TLR5, which is consistent with previous reports (Means et al., 2003; Uematsu et al., 2006) (Figure 3E, inset). Therefore it is likely that, in LP-DCs, flagellin recognition is mediated by both TLR5 and TLR11, possibly as a heterodimer, whereas in LP-M $\phi$ , TLR11 homodimers recognize flagellin. Therefore, although TLR11 and TLR5 both recognize flagellin and can form a heterodimer, heterodimer formation is clearly not a prerequisite for flagellin recognition, which is consistent with the lack of TLR11 in humans.

We had noted that TLR11 is upregulated in macrophages upon exposure to *S. Typhimurium* or flagellin (Figure S3B). Therefore, we decided to examine the expression of TLR5 and TLR11 in LP-M $\phi$  before and after infection with *S. Typhimurium*. Consistent with the in vitro data, we observed an increase in TLR11 mRNA upon infection. Surprisingly, TLR11 mRNA was upregulated in *tlr5*<sup>-/-</sup> macrophages both before and, to a greater extent, after



**Figure 3. Recognition of *S. Typhimurium* Flagellin by TLR5 and TLR11**

(A) WT and *tlr11*<sup>-/-</sup> mice (n = 5) were injected intraperitoneally with recombinant *S. Typhimurium* flagellin, and 2 hr later, serum IL-6 and IL-12 were measured by ELISA. \*p < 0.01.

(B) TLR5 and TLR11 were expressed in HEK293 cells for 48 hr, and TLR/flagellin complexes were formed by incubating cell lysates with His-tagged recombinant *S. Typhimurium* flagellin (1 μg/ml) overnight at 4°C. Binding was assessed by Ni-sepharose pull-down followed by western blotting with anti-Flag, anti-V5, and anti-His antibodies.



infection (Figures 3C and S3C). This suggests that, in *tlr5*<sup>-/-</sup> mice, the LP-M $\phi$  are activated, most likely indirectly, and as a result, the level of TLR11 is increased in these cells. Sorted CD11b<sup>+</sup> LP-M $\phi$  from *tlr5*<sup>-/-</sup> mice express increased levels of the activation markers CD86 and MHC class II after infection, similar to the WT mice, which is consistent with the activated status of these macrophages (Figure 3D). Consistent with enhanced TLR11 expression in *tlr5*<sup>-/-</sup> LP-M $\phi$ , these cells were hyperresponsive to heat-killed *S. Typhimurium* (HKS) (Figure 3E) and infection. Although there was a modest increase in basal cytokine production observed in the unstimulated LP-M $\phi$ , only the HKS-induced response was abrogated upon deletion of TLR11 (Figure 3E). Therefore, hyperresponsiveness of TLR5<sup>-/-</sup> LP-M $\phi$  to *S. Typhimurium* PAMPs required TLR11. These results suggest that increased TLR11 expression in TLR5 KO macrophages leads to their enhanced activation and increased recruitment of CD4 T cells to the lamina propria, thereby facilitating clearance of *Salmonella* from these mice. This model suggests that the TLR11 KO phenotype would be dominant during *S. Typhimurium* infection. Indeed, *tlr5*<sup>-/-</sup>/*tlr11*<sup>-/-</sup> mice exhibit enhanced lethality like *tlr11*<sup>-/-</sup> mice (Figure 3F). Arguably, the lethality of the double-KO mice and *S. Typhimurium* dissemination are further enhanced (Figure S3D), suggesting that loss of TLR11 may reveal a protective role for TLR5.

### TLR11 Knockout Mice Are Susceptible to Infection by *S. Typhi*

The vast majority of animal studies on *Salmonella* have been carried out by using *S. Typhimurium*, a *Salmonella enterica* serovar that causes a typhoid-like disease only in mice. In contrast, *S. Typhi* causes typhoid fever in humans but does not infect mice (Tsolis et al., 1999). This lack of a convenient small-animal model has prevented careful study of the immune response to *S. Typhi* (Raffatellu et al., 2008). As a result, there are significant outstanding questions regarding *S. Typhi* biology and, especially, the host immune response to this important pathogen. Consequently, development of effective vaccines against *S. Typhi* has been significantly hampered. Although oral *S. Typhi* vaccines exist, efficacy has been limited to ~70% (Levine et al., 1999, 2007); although more effective conjugate vaccines are available, these are ineffective in young children (Lin et al., 2001). Furthermore, the development of *Salmonella* spp. as potential vaccine vectors is also hindered by the inability to infect rodent models (Mestecky et al., 2008).

TLR11 is expressed in mice, but not humans, and we hypothesized that expression of TLR11 might be responsible for preventing infection by *S. Typhi*. Although *S. Typhi* is thought to modulate expression of flagellin in order to evade recognition

by the host, this occurs after engaging the epithelial barrier (Arricau et al., 1998; Winter et al., 2008). Thus, expression of TLR11 and recognition of *S. Typhi* at the epithelium in mice may be a crucial determinant of murine resistance to *S. Typhi*. To test this hypothesis, we orally administered *S. Typhi* to WT and *tlr11*<sup>-/-</sup> mice. As expected, WT mice were unaffected by *S. Typhi*. In contrast, all of the *tlr11*<sup>-/-</sup> mice were infected and displayed significant mortality (Figure 4A). Infection of *tlr11*<sup>-/-</sup>, but not WT, mice was accompanied by significant upregulation of serum IL-12 (Figure 4B) and TNF (Figure 4C), suggesting disseminated disease. Indeed, severe intestinal tissue damage with areas of complete epithelial breakdown was observed in *tlr11*<sup>-/-</sup> mice (Figures 4D and 4E). Furthermore, bacterial dissemination to various organs, along with high colony forming units (CFU) load, was observed in *tlr11*<sup>-/-</sup> mice (Figure 4F). We also examined the presence of cell death in spleen and liver sections in infected mice by using TUNEL staining and observed increased TUNEL-positive cells in sections from *tlr11*<sup>-/-</sup> mice (Figure 4G). Although previous studies have shown mild differences in TLR5 responses to *Salmonella* sp. flagellin, these results suggest that the lack of TLR11 in humans may be a more crucial determinant of *Salmonella* susceptibility. Thus, the host tropism of *S. Typhi* is restricted by a host-specific TLR.

Nontyphoidal *Salmonella* serovars (NTS) cause a very different disease compared to typhoidal *Salmonella* serovars, such as *S. Typhi* and *S. Paratyphi*. In humans, in whom NTS cause local gastroenteritis and diarrhea, these bacteria do not breach the epithelial barriers and are cleared by the mucosal immune system. In contrast, *S. Typhi* overcomes the mucosal barrier even in healthy adults and is disseminated to internal organs. A feature of typhoid infection in humans is its persistence, where the patient develops high fever for extended periods. We therefore wanted to test whether *tlr11*<sup>-/-</sup> mice infected with *S. Typhi* also developed fever. The pathogenic *S. Typhi* strain used in these studies is well studied and characterized (*S. Typhi*-Ty2 pathogenic strain from ATCC 700931) and expresses the Vi capsular antigen (Figure S4A), which is a major virulence factor for *S. Typhi*. *Tlr11*<sup>-/-</sup> mice infected with a relatively low dose (CFU 10<sup>6</sup>) of orally administered *S. Typhi* developed fever, whereas, as expected, the WT mice were uninfected (Figure S4B). Fever persisted for ~3 weeks before dissipating. In contrast, when the mice were infected with the normal, higher dose (CFU 5 × 10<sup>8</sup>) of *S. Typhi*, the TLR11 KOs developed fever but then developed sepsis around day 10 with hypothermia and death (Figure S4C). Stool samples from the infected mice revealed significantly increased levels of gross blood and bloody diarrhea in the *tlr11*<sup>-/-</sup> mice (Figure S4D). Careful examination of the ileum, colon, and caecum of the infected *tlr11*<sup>-/-</sup> mice showed

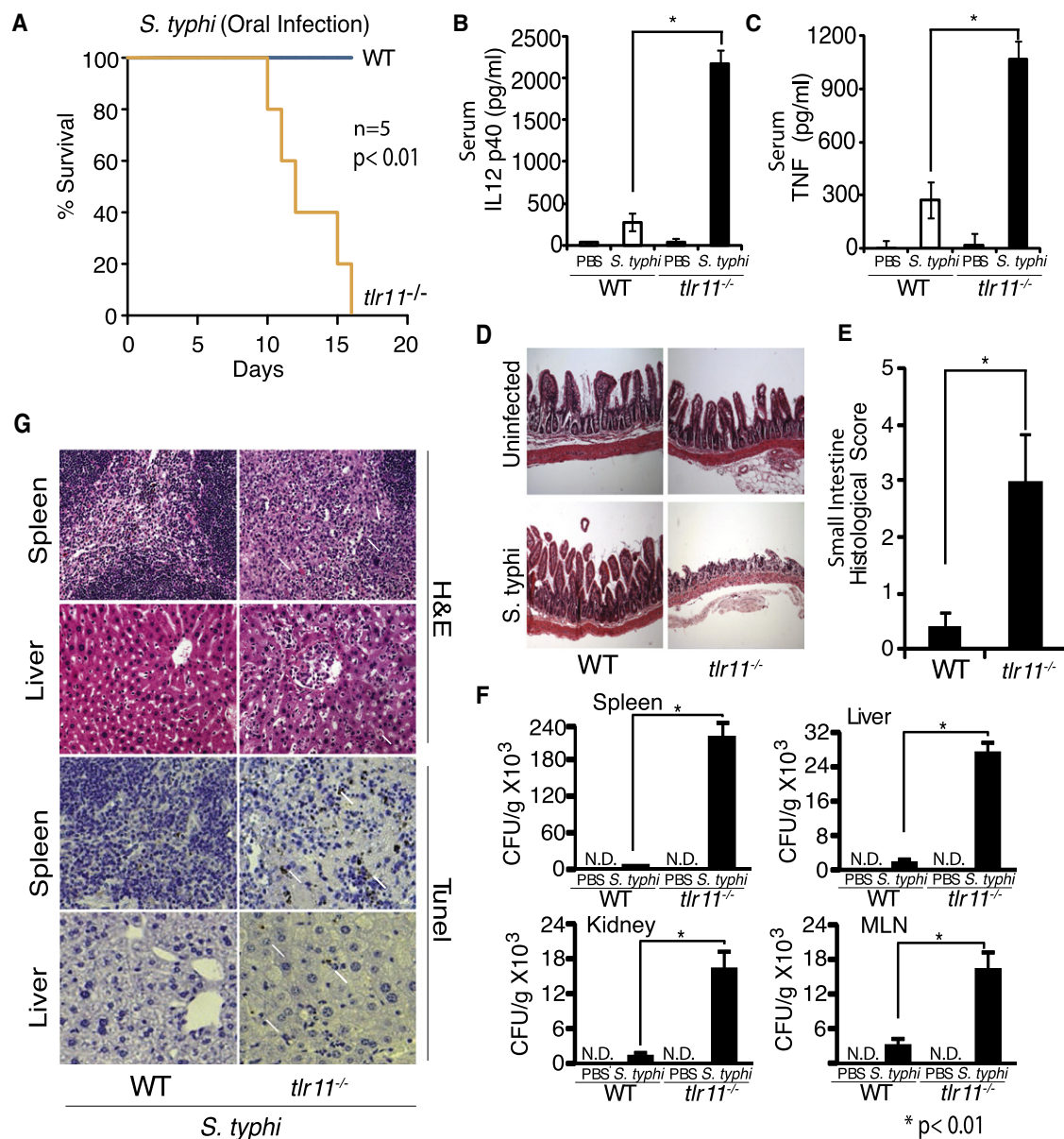
(C) Wild-type, *tlr11*<sup>-/-</sup>, and *tlr5*<sup>-/-</sup>/*tlr11*<sup>-/-</sup> mice were challenged with *S. Typhimurium*, and 5 days later, mice were sacrificed, and lamina propria macrophages were obtained by sorting (CD11c<sup>-</sup>CD11b<sup>+</sup> MHC-II<sup>+</sup>). RNA was prepared and analyzed for TLR5 and TLR11 expression by RT-PCR.

(D) Lamina propria cells from above were analyzed for macrophage activation markers (CD80, CD86, MHC-II, and CD40) by FACS. The blue traces are from infected mice.

(E) Lamina propria macrophages and DCs were sorted from WT mice and analyzed for TLR5 and TLR11 expression by RT-PCR (inset). Cells from WT, *tlr11*<sup>-/-</sup>, and *tlr5*<sup>-/-</sup>/*tlr11*<sup>-/-</sup> mice were stimulated in vitro with heat-killed *S. Typhimurium*, and IL-6 (upper graph) and IL-12 (lower graph) production was measured by ELISA.

(F) WT, *tlr11*<sup>-/-</sup>, and *tlr5*<sup>-/-</sup>/*tlr11*<sup>-/-</sup> mice (n = 8) were challenged with *S. Typhimurium*.

Related to Figure S3.



**Figure 4. *Tlr11*<sup>-/-</sup> Mice Are Susceptible to Oral Infection with *Salmonella Typhi***

(A) Survival of WT and *tlr11*<sup>-/-</sup> mice (n = 5 mice) orally infected with 10<sup>9</sup> CFU *S. Typhi* (S. Typhi-Ty2, ATCC 700931).

(B) Day 10 serum IL-12 p40 from mice orally infected with 10<sup>9</sup> CFU *S. Typhi* per mouse.

(C) ELISA analysis of serum TNF from mice orally infected with *S. Typhi* as in (B). \*p < 0.01.

(D and E) (D) Histology of orally infected WT and *tlr11*<sup>-/-</sup> mice was assessed on day 10 postinfection by using small intestine sections, and (E) histological score was plotted.

(F) *S. Typhi* dissemination detected by CFU per gram of tissue on LB plates from spleen, MLN, liver, and kidney.

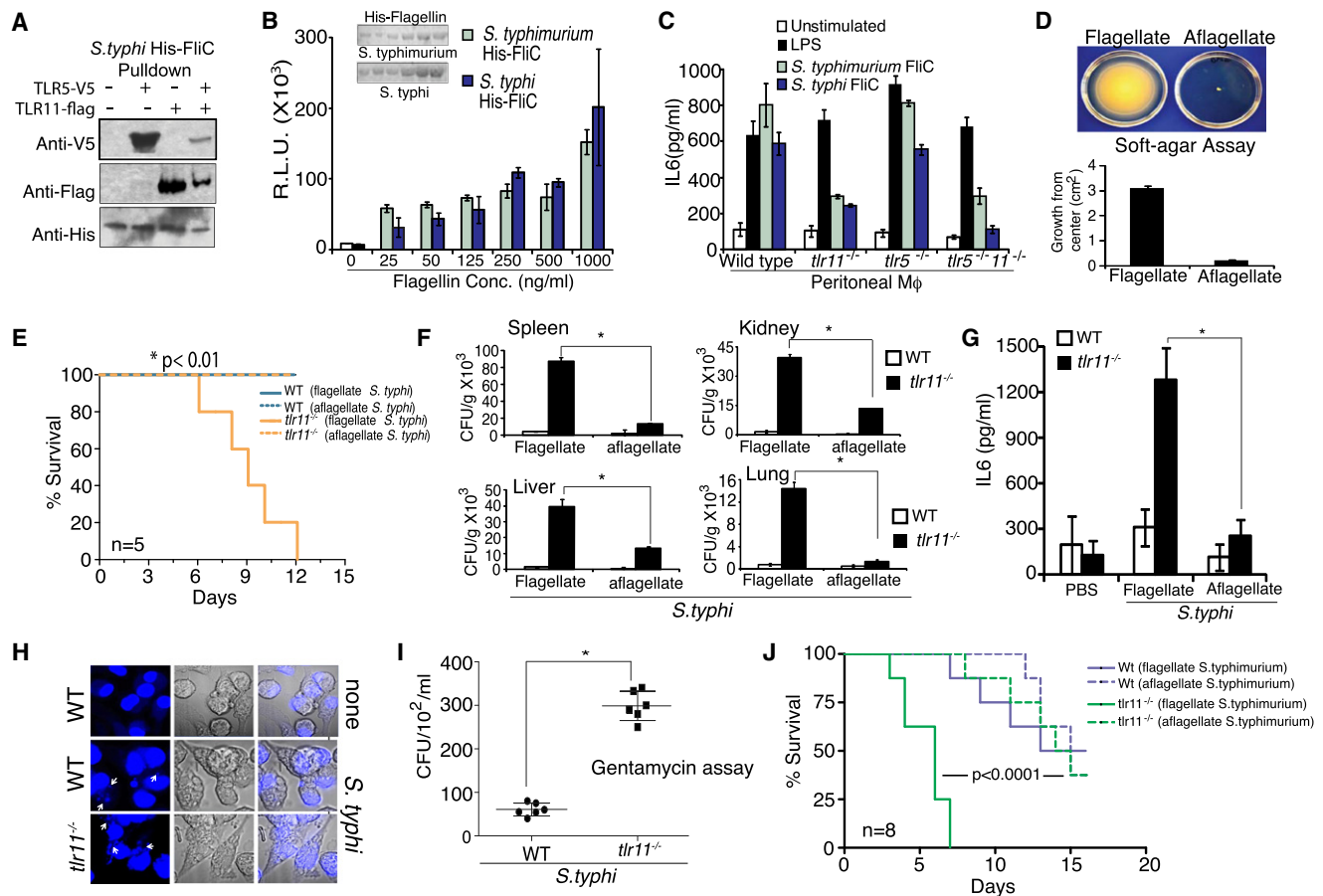
(G) H&E staining and tunel staining of tissues from day 10 postinfection with *S. Typhi*. The arrows indicate tunel-positive cells.

Related to Figures S4 and S5.

breaches in the epithelial layer that were likely responsible for the bleeding that was observed (Figures S5A and S5B). Histology from *tlr11*<sup>-/-</sup> demonstrated evidence of disseminated disease in spleen, kidney, and lung (Figures S5C and S5D).

During typhoid infection, the gallbladder is often colonized by *S. Typhi* and can serve as a reservoir in chronic carriers, the most

notorious example of which was the case of Typhoid Mary. Mice infected with *S. Typhimurium* can also show infection of the gallbladder (Menendez et al., 2009). We examined the gallbladders from *tlr11*<sup>-/-</sup> mice infected with *S. Typhi*. Seven days postinfection, we observed significant numbers of bacteria in the gallbladders, both microscopically and upon culturing



### Figure 5. *S. Typhi* Infection of *tlr11*<sup>-/-</sup> Mice Requires Flagellin

(A) TLR/flagellin complexes were formed by incubating cells expressing TLR11 or TLR5 with His-tagged recombinant *S. Typhi* flagellin. Binding was assessed by Ni-sepharose pull-down followed by western blotting with anti-Flag, anti-V5, and anti-His antibodies.

(B) *S. Typhimurium* and *S. Typhi* flagellin were cloned in pET-15b vector, and flagellin was purified by affinity chromatography (inset). RAW NF- $\kappa$ B luciferase cells were stimulated with increasing doses of flagellin and assayed for luciferase activity.

(C) WT, *tlr11*<sup>-/-</sup>, and *tlr5*<sup>-/-</sup>/*tlr11*<sup>-/-</sup> peritoneal macrophages were stimulated with flagellin (500 ng/ml), and supernatant IL6 was measured by ELISA.

(D) Aflagellate *S. Typhi* was obtained from the Salmonella Genome Stock Centre (SGSC; Calgary, Canada), examined for motility on soft agar plate, and grown in a humid chamber overnight at 37°C. Bacterial growth was measured from center and plotted.

(E) WT and *tlr11*<sup>-/-</sup> mice (n = 5) were orally infected with flagellate or aflagellate *Salmonella Typhi* (5 × 10<sup>8</sup> CFU).

(F) Mice were sacrificed at day 10, and tissue CFU in spleen, kidney, liver, and lung was measured by using LB-agar plates. \*p < 0.01.

(G) On day 10, serum cytokines were analyzed by ELISA.

(H) WT and *tlr11*<sup>-/-</sup> mouse peritoneal macrophages were infected with *S. Typhi* at an MOI of 10 for 6 hr and stained with DAPI (indicated by arrows).

(I) Macrophages infected for 2 hr were washed three times, placed in media with 100  $\mu$ g/ml gentamycin, and incubated for 4 hr. Macrophage lysates prepared with Triton X-100-containing media were plated on the LB agar plates to determine CFU.

(J) WT and *tlr11*<sup>-/-</sup> mice (n = 8) were orally infected with flagellate and aflagellate *Salmonella Typhimurium* (CFU-10<sup>8</sup>/mice).

Related to Figure S6.

the bacteria from the tissue (Figures S5E and S5F). Therefore, *tlr11*<sup>-/-</sup> mice infected with *S. Typhi* show many of the hallmarks of human typhoid.

### Infection of TLR11 Knockout Mice by *S. Typhi* Is Dependent on Flagellin

To test whether the flagellin from *S. Typhi* was also recognized by TLR11, we cloned and expressed a His-tagged form of flagellin from *S. Typhi*. In vitro pull-down experiments, performed in parallel with the *S. Typhimurium* flagellin pull-down shown in Figure 3B, we find that *S. Typhi* flagellin is capable

of binding both TLR5 and TLR11 similarly (Figure 5A). We purified flagellin from *S. Typhimurium* and *S. Typhi* by using nickel affinity columns followed by passage through a polymyxin B column to remove LPS (Figure 5B, inset) and tested their ability to stimulate NF- $\kappa$ B activity in the RAW 264.7-luciferase reporter cell line that does not express TLR5 (Figure 2B). Flagellins from both spp. were equivalent in the ability to stimulate NF- $\kappa$ B in these cells. To test the TLR dependence of flagellin recognition, we stimulated peritoneal macrophages from WT, *tlr11*<sup>-/-</sup>, *tlr5*<sup>-/-</sup>, and *tlr11*<sup>-/-</sup>/*tlr5*<sup>-/-</sup> mice and measured IL-6 production by ELISA. The level of IL-6 produced was dramatically reduced



in cells from *tlr11*<sup>-/-</sup> mice, but not from *tlr5*<sup>-/-</sup> mice (Figure 5C), which is consistent with the lack of significant expression of TLR5 in macrophages (Means et al., 2003; Uematsu et al., 2006).

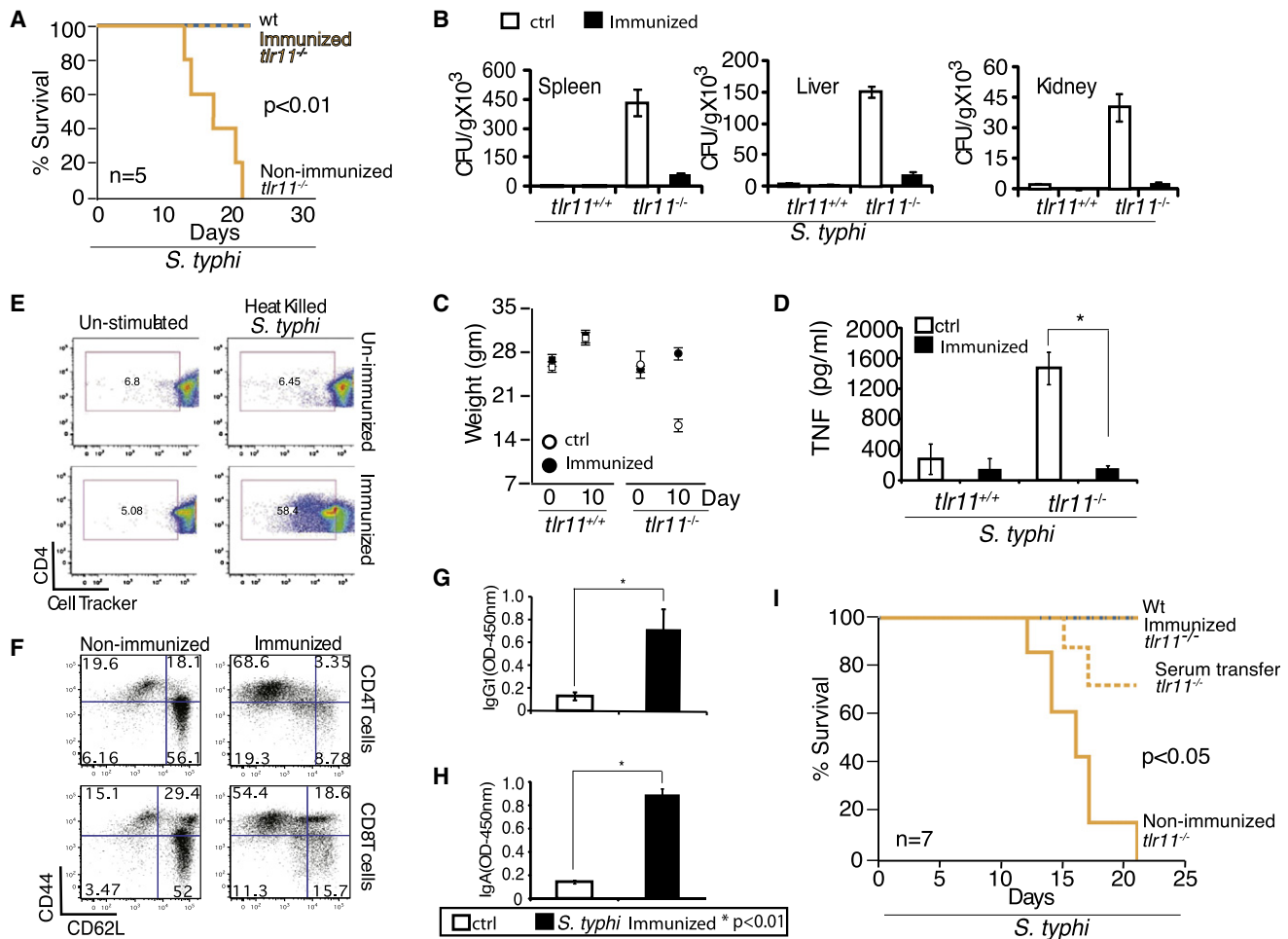
As flagellin from both *S. Typhi* and *S. Typhimurium* are recognized by TLR11, we wondered why mice would be resistant to only *S. Typhi*. TLR11 is absent in humans, and interestingly, similar to the situation in mice, *S. Typhi* does not infect a variety of other species that coincidentally also express TLR11. Sequencing efforts have revealed that *S. Typhi* has a significantly degraded genome that expresses far fewer virulence factors compared to *S. Typhimurium* (Parkhill et al., 2001). Hence, a possible explanation for the species-specific resistance to *S. Typhi* by TLR11 would be the unique dependence of *S. Typhi* on flagellin as a virulence factor. Consistent with such a hypothesis, a previous study found minimal effects of the loss of flagella on *S. Typhimurium* infection in mice, suggesting that other virulence factors were substituting for the absence of flagellin (Lockman and Curtiss, 1990; Schmitt et al., 2001). In contrast, aflagellate *S. Typhi* exhibited defects in epithelial cell invasion in vitro that were not solely due to lack of motility (Liu et al., 1988). To directly test the role of flagellin in the infection of mice by *S. Typhi* and *S. Typhimurium*, we obtained aflagellate versions of both strains. We first ensured that the aflagellate *S. Typhi* also expressed Vi antigen (Figure S4A). As expected, the motility of aflagellate *S. Typhi* on a soft agar assay was dramatically reduced (Figure 5D). We then compared the ability of flagellated and aflagellated *S. Typhi* to infect WT and *tlr11*<sup>-/-</sup> mice. Only *tlr11*<sup>-/-</sup> mice developed lethal infection with flagellated *S. Typhi*; however, these mice were unaffected by aflagellated *S. Typhi* (Figure 5E). We also characterized the mice infected with the aflagellated *S. Typhi* and observed reduced dissemination (Figure 5F) and cytokine production (Figure 5G). These data, with previous in vitro experiments (Liu et al., 1988), suggest a particular dependence of *S. Typhi* on flagellin as a key virulence factor in vivo.

Mice are ordinarily resistant to *S. Typhi*, even when introduced by intravenous or intraperitoneal inoculation. The transplantation of mice with human immune cells can render mice susceptible to *S. Typhi* infection by intraperitoneal inoculation, suggesting that the host specificity of *S. Typhi* also results from a requirement for human mononuclear cells (Firoz Mian et al., 2011; Libby et al., 2010; Song et al., 2010). Our studies suggest that TLR11 is critical for preventing infection by *Salmonella Typhi* following the normal (oral) transmission route. However, to test directly whether TLR11 also affects the ability of the bacteria to infect macrophages, we isolated peritoneal macrophages, which lack TLR5, from wild-type and TLR11 KO mice and infected them with *S. Typhi*. The TLR11 KO macrophages were infected with significantly greater numbers of bacteria as determined by direct microscopic analysis (Figure 5H). *S. Typhi* in infected *tlr11*<sup>-/-</sup> macrophages was viable and could be recovered from infected macrophages. Infected macrophages were incubated in gentamycin-containing media after infection to kill noninternalized bacteria, followed by separating and washing the cells and performing CFU assays (Figure 5I). We also compared the ability of flagellated and aflagellated *S. Typhi* to infect TLR11 KO peritoneal macrophages in culture

by using the same microscopic and gentamycin culture assays, and we observed a clear dependence on flagellin for *S. Typhi* infection of mouse macrophages (Figure S6A). Finally, we wanted to test the role of TLR11 when mice were infected by intraperitoneal injection of *S. Typhi*. It is well known that the restriction against *S. Typhi* in mice is not due to a barrier effect, as mice are even resistant to intraperitoneal infection (Collins and Carter, 1978). Recent chimeric humanized mouse models demonstrate that the host restriction in bone-marrow-derived cells applies to systemically administered *S. Typhi* (Firoz Mian et al., 2011; Libby et al., 2010; Song et al., 2010). Consistent with the infection experiments in culture, *S. Typhi* did not cause infection of WT mice, whereas the absence of TLR11 led to lethal systemic infection, even with a low-dose infection (10<sup>4</sup> CFU per animal) with dissemination of the bacteria to different organs (Figures S6C and S6D). In contrast to *S. Typhi*, when oral infections were performed with flagellated and aflagellated *S. Typhimurium*, we observed significant and similar lethality in WT and *tlr11*<sup>-/-</sup> mice with the aflagellated *S. Typhimurium* (Figure 5J). These results suggest that other virulence factors present in *S. Typhimurium*, but not in *S. Typhi*, mitigate the requirement for flagellin and offer a plausible explanation for why *S. Typhi*, but not *S. Typhimurium*, exhibits strict human host restriction.

### TLR11 KO Mice Can Be Immunized against Infection by *S. Typhi*

Analysis of the *tlr11*<sup>-/-</sup> mice infected with *S. Typhi* suggests that TLR11 provides effective immune responses at the intestinal epithelial barrier, preventing entry and subsequent dissemination of the bacteria. As *S. Typhi* are gram-negative bacteria that express numerous other PAMPs and as the immune system of these mice is not otherwise compromised, we assumed that adaptive immune responses to injected *S. Typhi* would be maintained. The more delayed disease course in *tlr11*<sup>-/-</sup> mice compared with recent humanized mouse models (Libby et al., 2010) is consistent with *tlr11*<sup>-/-</sup> mice having otherwise normal immune systems. The recovery of mice that are given low-dose oral infection and exhibit a typhoid-like disease course further distinguishes the *tlr11*<sup>-/-</sup> mice as an *S. Typhi* disease model. These findings led us to speculate that *tlr11*<sup>-/-</sup> mice would be a useful model for vaccine development. We therefore immunized wild-type and *tlr11*<sup>-/-</sup> mice by injecting heat-killed *S. Typhi* intraperitoneally, with the first injection followed by four booster injections at 4 day intervals. As a control, the unimmunized mice were injected with PBS. Fourteen days after the final booster injection (i.e., 30 days after the first injection), the mice were given live *S. Typhi* (CFU 5 × 10<sup>8</sup>) orally and monitored for 3 weeks. Whereas all unimmunized *tlr11*<sup>-/-</sup> mice died by day 20 of infection, immunization provided complete protection against *S. Typhi* infection in *tlr11*<sup>-/-</sup> mice (Figure 6A). There was very little dissemination of *S. Typhi*, and immunized *tlr11*<sup>-/-</sup> mice gained weight similar to WT mice (Figures 6B and 6C). Immunization of *tlr11*<sup>-/-</sup> mice also reduced cytokine responses to levels that were comparable to those of WT mice (Figure 6D). We examined the antibodies in the immunized animals and detected elevated IgG1 and IgA (Figures 6G and 6H). Proliferation of CD4<sup>+</sup> T cells was augmented (Figure 6E), whereas the number of CD62L low/CD44 high cells was significantly



**Figure 6. Generation of Protective Immunity in *tlr11*<sup>-/-</sup> Mice by Immunization**

(A) WT and *tlr11*<sup>-/-</sup> mice immunized with or without  $10^8$  CFU equivalents of heat-killed *S. Typhi* (HKS) at days -30, -26, -22, -18, and -14 were challenged by oral administration of  $5 \times 10^8$  CFU live *S. Typhi* on day 0 ( $n = 5$  per group).

(B) Day 10 postinfection CFU from spleen, liver, and kidney homogenates measured by plating on LB agar plates.

(C) Weight of WT and *tlr11*<sup>-/-</sup> mice injected with or without heat-killed *S. Typhi* and subsequently orally challenged with live *S. Typhi* as in (A) ( $n = 5$ ).

(D) TNF ELISA performed from serum samples collected at day 10 postinfection of HKS-immunized versus control nonimmunized mice as in (A). \* $p < 0.01$ .

(E) Proliferation of Cell-Tracker-labeled CD4 T cells cultured in vitro for 48 hr with antigen-presenting cells simulated with HKS was analyzed by FACS.

(F) Memory phenotype of splenic T cells from *tlr11*<sup>-/-</sup> mice, either nonimmunized or HKS immunized, was assessed by analysis of CD44 and CD62L by FACS.

(G and H) Serum IgG1 and IgA measured by ELISA from uninfected animals immunized as in (A).

(I) Survival of WT and *tlr11*<sup>-/-</sup> mice infected with *S. Typhi*. The *tlr11*<sup>-/-</sup> mice were either nonimmunized or HKS immunized or were injected with serum (100  $\mu$ l) from immunized WT mice before challenge with live, orally administered *S. Typhi* (CFU  $5 \times 10^8$ ).

increased in immunized animals (Figure 6F), suggesting development of strong memory responses that are critical for successful vaccination.

Finally, to see whether generated immunity to *S. Typhi* could be transferred to an unimmunized TLR11 KO, we isolated serum from the WT mice immunized with heat-killed *S. Typhi* and transferred it to uninfected TLR11 KO mice. The recipient mice were then infected with live *S. Typhi*, and we observed significant protection against lethality in these mice (Figure 6I). This result strongly suggests that the TLR11 KO mice are likely to be an excellent model to study protective immune responses to *S. Typhi*, and hence facilitate efforts to develop more effective human vaccines.

## DISCUSSION

In this study, we report that the nonhuman TLR, TLR11, recognizes flagellin and helps prevent oral *Salmonella* spp. infection and dissemination in mice. Interestingly, *S. Typhi*, an obligate human pathogen that does not infect WT mice, can efficiently infect *tlr11*<sup>-/-</sup> mice. This represents a clear demonstration of pathogen host restriction determined by species-restricted TLR expression. As such, *tlr11*<sup>-/-</sup> mice resemble mice with a humanized innate immune system, at least with respect to recognition of *Salmonella* flagellin. Thus, absence of TLR11 abolishes the innate immunity that normally prevents *S. Typhi* infection. This restriction likely occurs at the level of tissue-resident

macrophages, which express high levels of TLR11 and are nonpermissive for *S. Typhi* infection. Given that TLR11 is also expressed in small intestinal epithelial cells as well as in dendritic cells, it is possible that TLR11 acts to both promote barrier responses against *Salmonella* spp. and to augment systemic adaptive and innate responses after the epithelial barrier is breached. The relative contribution of various TLR11-expressing cellular compartments remains to be fully explored. Nevertheless, in the absence of TLR11, *Salmonella* spp. disseminate more readily to other organs, including gallbladder, spleen, liver, and kidney. Upon infection with a high inoculum, there is significant epithelial damage, and a robust disseminated infection is established. *S. Typhi* PAMPs detected by TLRs such as TLR2, TLR4, and TLR5 are likely to lead to systemic cytokine production and septic shock.

In the context of a low-dose infection of otherwise immunocompetent *tlr11*<sup>-/-</sup> mice, a more classical resolving typhoid disease is observed. In this context, other aspects of the innate immune system are likely to be protective. Hence, the *tlr11*<sup>-/-</sup> mice should allow the contribution of TLR5 and other components of the innate and adaptive immune response to be addressed in a small-animal model that more closely mimics the complement of innate *Salmonella* sensors present in humans. It is possible that, as for *S. Typhimurium* infection, in which loss of TLR5 further sensitizes TLR11 KO animals, *tlr11*<sup>-/-</sup> mice may reveal that TLR5 is a relevant sensor of *S. Typhi*. The ability of *tlr11*<sup>-/-</sup> mice to respond normally to internalized bacteria means that they can be immunized by injection of heat-killed *S. Typhi*. The protective immunity generated in immunized *tlr11*<sup>-/-</sup> mice is highly effective and completely blocks infection upon subsequent oral challenge with live bacteria. The ability to infect and successfully vaccinate the TLR11 KO means that these mice represent a mouse model for *S. Typhi* infection and should significantly facilitate future research into both the pathobiology of this important pathogen and the development of effective vaccination strategies against *S. Typhi* in humans.

Toll-like receptors have emerged as critical sensory mechanisms for recognizing and responding to infectious microbes. Although most TLRs are shared between humans and other species such as mice, a subclass that includes TLR11, 12, and 13 is not expressed in humans. TLR11 is expressed in many species such as birds, dogs, and cows but is absent in humans. Previous studies have shown that TLR11 recognizes profilin, a protein ligand from the apicomplexan parasite *Toxoplasma gondii*, and plays a critical role in the mounting of innate immune responses to this organism (Yarovinsky et al., 2005). In addition, TLR11 was also shown to specifically respond to uropathogenic *E. coli* strains that typically infect the urogenital tract, causing urinary tract infections (Zhang et al., 2004). The expression of TLR11 in epithelial cells, as well as macrophages and dendritic cells, likely explains its involvement in mediating responses to these different pathogens. The studies presented in this report show that TLR11 recognizes flagellin from both the UPEC strain 8NU and *Salmonella* spp. In this regard, it is particularly interesting to note that there are several important flagellated enteropathogenic bacteria—for example, enteropathogenic *E. coli* (Mohawk and O'Brien, 2011) and *Campylobacter jejuni* (Young et al., 2007)—for which mouse models do not recapitulate

human disease. Instead, many studies must be carried out by using immunocompromised neonatal mice, colonization of germ-free and streptomycin-treated mice, massive bacterial doses, nonphysiological routes of administration, or more limited models such as the rabbit ileal loop or in vitro models. Although these and other approaches have proven extremely useful, they also have significant limitations, most notably for the study of host immunity and for vaccine development. Unlike other animal models in which conserved aspects of the innate immune response have been removed, such as MyD88- or TLR4-deficient mice, the *tlr11*<sup>-/-</sup> mice maintain expression of all conserved components of the innate and adaptive immune system. As such, we speculate that *tlr11*<sup>-/-</sup> mice will be a useful animal model to study the microbiology and immunology of a wide variety of flagellated human pathogens and will also prove useful in vaccine development.

## EXPERIMENTAL PROCEDURES

### Experimental Animals

*Tlr11*<sup>-/-</sup> mice (Zhang et al., 2004) were backcrossed to the C57/BL6 background (Jackson Laboratories, Bar Harbor, ME) for more than ten generations. Mice used in experiments were generated by crossing *tlr11*<sup>-/+</sup> mice. Many experiments utilized WT and KO littermates, whereas in other experiments, mice produced by crossing TLR11 KO males and females and WT males and females, obtained from the first breeding, were used. Animals used for experiments were age and sex matched and were bred and housed in specific pathogen-free conditions (Yale University and Columbia University). All mouse protocols were approved by IACUC, Yale University, and Columbia University.

### Reagents and Antibodies

LPS 0111:B4 was from Sigma (St. Louis, MO). Recombinant flagellin produced in BL21 was purified by His-affinity chromatography and polymyxin B (Detoxi Gel; Thermo). Purified flagellin from mammalian cells was kindly provided by Dr. Andrew Gewirtz (Emory University; McSorley et al., 2002). Bacterial strains were *S. Typhi* (ATCC-700931), *Salmonella* Typhimurium (SL1344), and GFP-*Salmonella* strain (SL1344). Aflagellate *S. Typhimurium* (SL-1344; Aflagellate fliC fliB::Tn10 > fliB5001::MudJ) was from Dr. Louise Teel (Uniformed Services University of the Health Sciences). Aflagellate *S. Typhi* Ty2 [SA4717 (fliC::Tn10—Fli(-))] was from the *Salmonella* Genetic Stock Center (Calgary, Canada). Uropathogenic *E. coli* 8NU was from Dr. D. Klumpp (Northwestern University, IL).

### Purification of the *S. Typhimurium* TLR11 Ligand

*S. Typhimurium* (SL1344) was grown overnight at 37°C without shaking, collected by centrifugation, washed once in 10 mM Tris (pH 8), and homogenized in a Waring Blender for 5 × 10 s followed by heating at 60°C for 30 min. The resulting product was centrifuged at 10,000 ×g for 30 min, and the supernatant was ultracentrifuged at 100,000 ×g for 60 min. The resulting supernatant was treated with polymyxin B (Thermo/Pierce), applied to a DEAE Sepharose column, and eluted by step elution with 250 and 500 mM NaCl. Dialyzed fractions were assessed by NF-κB luciferase reporter assay, and fractions of interest were pooled and subjected to CM Sepharose chromatography. Fractions containing peak activity (flow through) were applied to Mono Q column and eluted with a linear 50–500 mM NaCl gradient (AKTA, GE biosciences). Fractions containing stimulatory activity were resolved by SDS-PAGE and visualized by Coomassie. A single band coeluting with the TLR11 stimulatory activity was excised and subjected to in-gel tryptic digest and MS analysis (Columbia University Protein Core Facility).

### mRNA Expression

Total RNA was isolated using Trizol (Invitrogen), and 1 µg of RNA was used for RT. One-tenth of this reaction was used as a template for PCR amplification

with Taq polymerase for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Primer sequences are in the [Supplemental Information](#).

### In Situ Hybridization

TLR11 cDNA fragments used as probes were amplified by using specific primers ([Supplemental Information](#)) to create a 450 bp fragment and subcloned into the pcDNA3 vector. Sense probes were prepared after linearization with EcoRI and transcription with SP6 polymerase; antisense probes were prepared after linearization with XbaI and transcription with T7 polymerase. In situ hybridization was performed as described previously ([Zhang et al., 2004](#)).

### NF-κB Luciferase Assays

RAW264.7 cells stably transfected with a NF-κB luciferase reporter construct pBILX-luc (Raw-κB-luc) were plated and, after 24 hr, were stimulated with 20 ng/ml of LPS or 1 μg/ml of flagellin for 6 hr. Luciferase activity was measured according to the manufacturer's instructions (Promega).

### Flow Cytometry and Cell Isolation

Cells were stained by using the antibodies described (e-Biosciences or BD) and analyzed on a BD LSR-II. Analysis was performed with FlowJo software. For cell proliferation, splenocytes were plated in 96-well plates, stimulated with heat-killed *Salmonella* for 72 hr, and analyzed for cell proliferation by using Cell Tracker Red (Invitrogen) according to the manufacturer's instructions. Thioglycollate-elicited macrophages ([Rao et al., 2010](#)) and lamina propria cells ([Denning et al., 2007](#)) were isolated from mice under sterile conditions by using established protocols (see [Supplemental Information](#)). Lamina propria cells were sorted by using a FACS ARIA at the Microbiology and Immunology Core Facility, Columbia University.

### ELISA Experiments

For in vitro ELISAs, peritoneal macrophages were cultured in RPMI1640 10% FBS, and TNF, IL-12, and IL-6 were measured. For in vivo ELISAs, WT and *tlr11*<sup>-/-</sup> mice (n = 5) were injected i.p. with 30 μg of recombinant flagellin and bled at 2 hr, and serum IL-6 and IL-12p40 levels were measured. To assess cytokine production during infection, the same strains of mice were orally infected with *S. Typhimurium* or *S. Typhi*, surviving animals were bled, and serum IL-12p40 and TNF, IL6, IgG1, or IgA levels were assessed by ELISA on d5 postinfection for *S. Typhimurium* and day 8–10 postinfection for *S. Typhi*. ELISA kits were from BD Bioscience (San Jose, CA).

### In Vivo Infection and Immunization

Wild-type and *tlr11*<sup>-/-</sup> mice were orally infected with *S. Typhimurium* (10<sup>8</sup>) or *Salmonella Typhi* (5 × 10<sup>8</sup> or 5 × 10<sup>9</sup>) using a gavage needle unless otherwise indicated. For immunization, WT and *tlr11*<sup>-/-</sup> mice were immunized by i.p. injection of heat-killed *S. Typhi* (10<sup>8</sup> CFU equivalents per animal) five times at 4 day intervals and challenged orally with live *S. Typhi* at 14 days after the last immunization. Survival was monitored by daily observation, and Kaplan Meier survival graphs were generated (Prism, GraphPad). n = 5 mice were taken for each condition unless otherwise indicated; significance is as given and was calculated in Prism.

### Histology

Tissues were harvested and then sectioned and H&E stained at Yale or Columbia histology core facilities. Images were captured by using a Zeiss Imager-M2. Tissue histology sections were examined, and histological grading was performed by a GI pathologist at Yale University. Sections were scored as follows: 0, normal; 1, normal with focal epithelial loss; 2, normal with mild surface epithelial loss; 3, partial necrosis and diffuse epithelial loss; 4, total necrosis.

### CFU

Bacterial burden was measured as follows: tissues were harvested and homogenized at 1 gm per ml equivalent in LB and serially diluted 1:10 in LB, and 100 μl was plated on LB plates. For *S. Typhimurium* CFU determination, LB plates were supplemented with streptomycin. After 16 hr, colonies were

counted; data are presented as mean ±SD of triplicate samples and are representative of at least three independent experiments.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2012.08.042>.

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